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Attaching redox proteins onto electrode surfaces by bis-silane

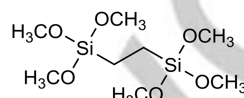
Stanislav Trashin[a], Mats de Jong[a], Vera Meynen[b], Sylvia Dewilde[c], Karolien De Wael[a]*

Abstract: Immobilization of redox proteins on electrode surfaces is of special interest for mechanistic studies and applications because of a well-controlled redox state of protein molecules by a polarized electrode and fast electron transfer kinetics, free from diffusion limitation. Here, bis-organosilane (1,2-bis(trimethoxysilyl)ethane) was applied as a fresh solution in a pH 7 phosphate buffer without use of any organic solvent, sol-gel or mesoporous bulk matrix. A short aging period of 30 minutes before deposition on the electrodes was optimal for the immobilization of proteins. Three redox proteins (cytochrome c, neuroglobin and GLB-12) were confined to the gold surface of electrodes with high coverages and stability, indicating that the suggested technique is simple, efficient and generic in nature.

Redox proteins such as cytochrome c (Cyt c) and globins play crucial roles throughout the life-cycle of cells, although their mechanisms of action are often not well understood.[1] Direct electrochemical studies of these proteins can provide an accurate determination of their formal potentials and facilitate investigations into their biochemical role, as well as possible applications in biosensors and/or biofuel cells.[2]

Extensive research investigations into human neuroglobin (NGB) have been ongoing for 15 years, yet its functions are still not fully understood. Moreover, the natural enzymatic system that is able to reduce NGB from its ferric form to the ferrous form *in vivo* is yet unknown.[1a, 3] Therefore assay studies of NGB rely on artificial reducing systems, which are either too slow or interfere with the oxygenated form of the protein. Alternatively, a polarized electrode can be employed as an efficient reductant, which is chemically orthogonal to the following reaction of ferrous NGB with its substrates. GLB-12 from *Caenorhabditis elegans* is a newly discovered member of the globin family that participates in reproduction by using its redox chemistry.[4] Electrochemical techniques offer important possibilities in regard to the detailed study of these and other recently discovered globins. Hence, there is an urge on a simple, universal but efficient method for confining redox proteins to electrodes which would not alter their active sites.

The main idea that underpins the presented immobilization procedure is the use of an ethane-bridged-bis-silane cross-linker in a monomeric or short oligomeric states, which are expected to be formed from a precursor in water prior to sol formation. Methoxy-substituted bridged silane (BTMSE, Scheme 1) was taken as the precursor. BTMSE appears to be a good candidate because it is commercially available, adheres well to hydroxyl-containing surfaces[5], and can form stable in water linear oligomers.[5b, 6] Moreover, the ethane-bridged-silane is probably long enough to play a role as a gentle cross-linker, while tetra-methoxysilane or alkyltrimethoxysilanes are simply too short for that.



Scheme 1. 1,2-bis(trimethoxysilyl)ethane (BTMSE).

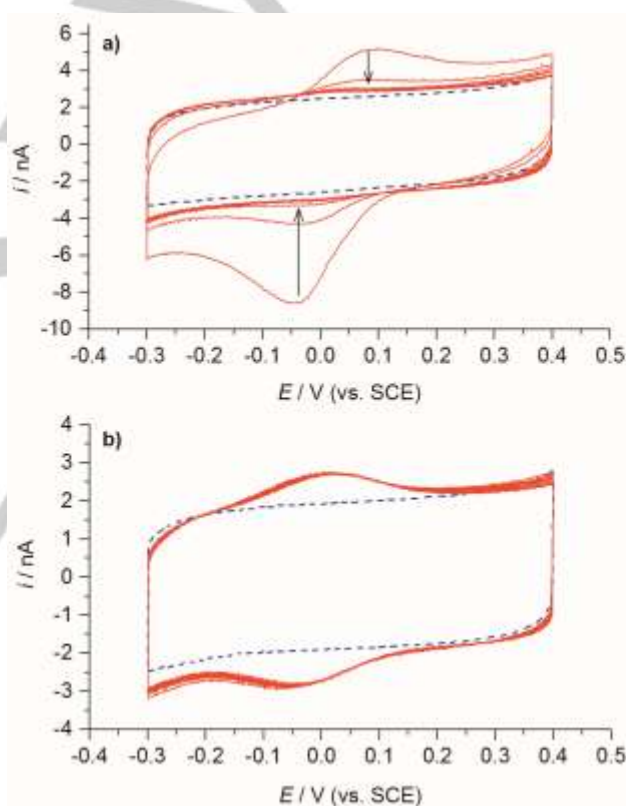


Figure 1. Behavior of physically adsorbed Cyt c (a) and surface-confined Cyt c with BTMSE (b). Dashed lines denote the background CV. Scan rate, 20 mV/s; 50 mM pH 7 phosphate buffer. Cycles 1-5, 10, 15, 20, 25 are shown. Arrows show direction of change.

In the conditions used here, the bis-organosilane was easily dissolved in phosphate buffer (pH 7) after one-two minutes of vortexing which resulted in a completely clear solution. The hydrolysis kinetics was assessed using ^1H NMR in D_2O medium (Supporting information). In first 20-30 min one methoxy-group, and after 60 min at least two methoxy-groups were hydrolyzed. Full hydrolysis in these conditions took about 6 hours. From this moment a suppression of the integral signal from the protons in the ethane bridge was observed indicating about formation of

[a] Dr. S. Trashin, M. de Jong, Prof. K. De Wael
AXES Research Group, Department of Chemistry
University of Antwerp
Groenenborgerlaan 171, 2010 Antwerpen, Belgium
E-mail: stanislav.trashin@uantwerpen.be and karolien.dewael@uantwerpen.be

[b] Prof. V. Meynen
Laboratory of Adsorption and Catalysis (LADCA), Department of Chemistry
University of Antwerp
Universiteitsplein 1, 2610 Wilrijk, Belgium

[b] Prof. S. Dewilde
PPES Research Group, Department of Biomedical Sciences
University of Antwerp
Universiteitsplein 1, 2610 Wilrijk, Belgium

Supporting information for this article is given via a link at the end of the document.

large oligomers and early stages of sol formation. A tenfold decrease was found in the integrated signal after 24 hours of aging but no sign of precipitate or gel formation was noticed. Gelation in 10 mM and 20 mM BTMSE solution in these conditions progressed relatively slow. The solutions formed a weak gel after seven days of aging at room temperature. More concentrated 0.1 M solutions of BTMSE prepared in the same buffer were stable for at least six hours, but gelation occurred when left overnight. In our procedure we used freshly prepared solutions of the BTMSE precursor with a final concentration of 10 mM, which obviously could not form any sol-gel during the time-scale required for the electrode preparation procedure. Thus, this technique must be distinguished from those using sol-gels or mesoporous silica matrix.[7]

To investigate the effect of BTMSE on the immobilization of proteins, we adsorbed Cyt c as a model protein with and without BTMSE on mercaptohexanol pre-modified gold electrodes and tested its electrochemical performance (Figure 1). When Cyt c was adsorbed alone onto the electrode, the peak current, linked to the redox chemistry of the heme unit, continuously decreased during CV (cyclic voltammetry) measurements, which is indicative of fast desorption and diffusion of the protein from the surface to the bulk. When the mixture of the protein and the bis-organosilane was deposited onto the surface, repetitive CV cycles showed a stable pair of bell-shaped redox peaks (Figure 1B) corresponding to an average surface coverage of 3.6 ± 1.1 pmol/cm^2 (24 ± 8 % of the monolayer from the maximal coverage of 15 pmol/cm^2 or 11 nm^2 per a Cyt c molecule).[8] It is clear from these results that BTMSE retained the protein on the surface, making it possible to continuously observe its direct oxidation/reduction.

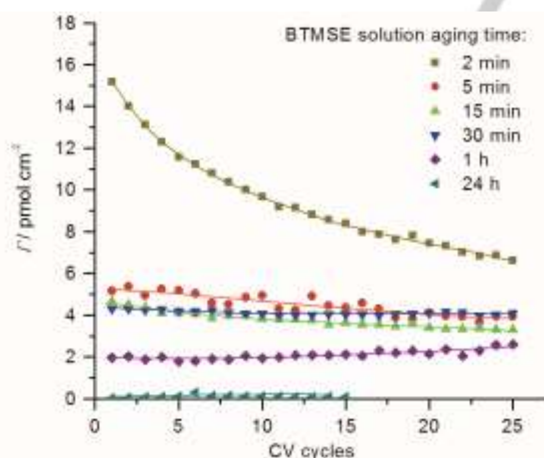


Figure 2. Effect of the aging time of the BTMSE solution on the protein coverage and stability of the prepared electrodes under continuous CV measurements.

One parameter of importance when designing this system for optimum performance was the protein concentration in the immobilization solution. In this study, concentration of 10 – 20 μM resulted in both a high surface coverage and an appropriate retention stability. A higher concentration of Cyt c in the solution caused poor stability and reproducibility in the adsorbed layer as well as slowing the electron transfer kinetics, leading to increased peak separation. This effect is probably related to the formation of protein multilayers and leak of the protein through the tenuous bridged oligo-organosilane film.

An important parameter that affects the performances of the protein-bis-organosilane film is the aging time of the bis-organosilane solution. When BTMSE is introduced into the buffer, two processes begin, hydrolysis and condensation. Unfortunately, hydrolysis kinetics are reported only for the ethoxy-precursor,[5a, 6, 9] while methoxy-groups are known to hydrolyze faster.[10] So we expect that after vortexing when the solution becomes completely clear, the precursor is at least partially hydrolyzed forming soluble hydroxy-bis-organosilane. Further progress is assumed to occur during the aging step and also during the drying of the protein-bis-organosilane mixture on the electrode surface, which takes an additional 60 minutes. We studied the effects of the BTMSE solution aging time (20 mM in 50 mM phosphate buffer, pH 7.0) before the solution was mixed in a 1:1 ratio with 30 μM Cyt c and deposited on the electrodes. The electrodes were tested under continuous potential cycling over approximately 20 minutes, and the results are shown in Figure 2.

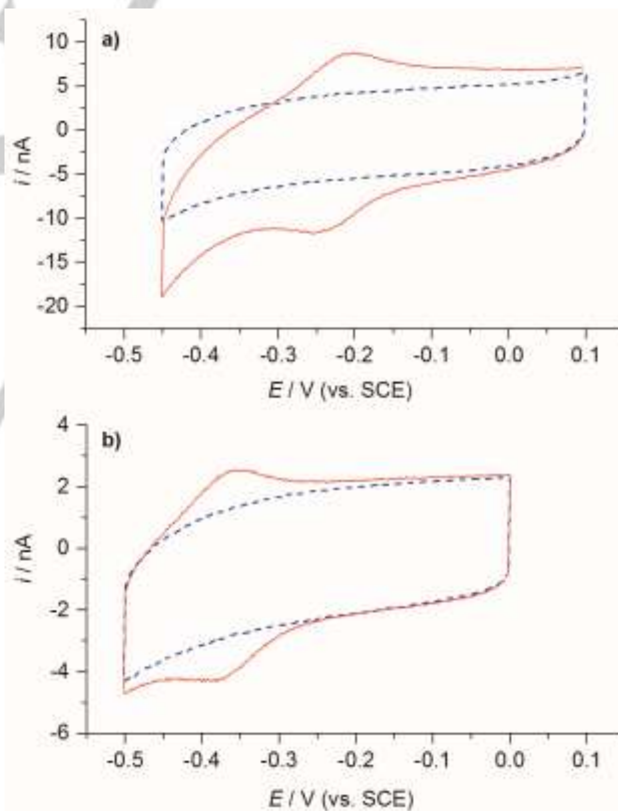


Figure 3. CV of GLB-12 (a) and human neuroglobin, NGB (b) immobilised with BTMSE. Dashed lines depict background CV. Scan rate, 20 mV/s; 50 mM pH 7 phosphate buffer; stable CV cycles in oxygen-free conditions.

The freshly prepared BTMSE solution gave relatively high but unstable protein surface coverage. Notably, the stability was still much better when compared to simple physisorption (Figure 1A), for which a rapidly diminishing peak could be observed over

only three cycles of CV. After maturing of the bis-organosilane solution for 5 to 30 minutes, the film surface coverage was approximately 4 pmol/cm^2, and noticeably improved stability was observed. However, for one hour of aging the protein coverage decreased to about half that seen for 30 minutes aging period, although a slight rise in the coverage was observed upon continued CV. Finally, after 24 hours aging at room temperature, only minor protein surface coverage remained while the BTMSE solution was still clear, without signs of precipitation or gelation. The decrease is probably related to hindering the contact between the protein and the electrode either because of shielding of the electrode surface by large silane oligomers or entrapment of the protein inside of non-conductive material. To conclude, 30 minutes of bis-organosilane solution aging is ideal for optimal immobilization.

Immobilized NGB (17 kDa; pI = 5.4) and GLB-12 (31.5 kDa; pI = 8.8) behaved similarly to Cyt c, leading to clear and intense redox peaks when they were immobilized along with BTMSE (Figure 3), while only minor and quickly disappearing peaks were observed for the proteins that were simply physisorbed onto the electrodes. The coverage calculated from the peak area was 54 ± 14 % and 13.3 ± 0.3 % of the monolayer for GLB-12 and NGB, respectively, using the same reference value of the maximal coverage as for Cyt c.

The linear dependence of the peak current on the scan rate (Figure S2, S3) suggests surface-confined behavior of the proteins, while a small gap between the oxidation and reduction peaks (Figure S4), which is virtually independent from the scan rate up till 2 V/s, reveals fast electron transfer kinetics with k_s > 100 s^{-1}. The nature of the gap is not clear, and has been previously reported as non-ideal behavior not related to electron transfer kinetics.[11]

The formal potential for immobilized Cyt c ($E^{\circ\prime}$= −0.011 ± 0.005 V) was about 50 mV more negative than the value for Cyt c in solution ($E^{\circ\prime}$= 0.036 ± 0.004 V). This shift is similar to a previously reported potential shift of 70 and 90 mV for electrostatically and covalently immobilized Cyt c respectively.[12] The shift is associated with some changes in surface charge distribution in Cyt c and was also found in Cyt c bound to mitochondrial membranes, liposomes and some proteins.[13] The formal potentials of NGB (-0.371 ± 0.006 V) and GLB-12 (-0.228 ± 0.003 V) in the solution were the same as those obtained for immobilized proteins, which suggests that their heme-pockets were intact after immobilization.

We hypothesize that ethane-bridged-hydroxy-silane and its oligomers work as gentle cross-linkers forming chemical bonds between protein molecules and the surface. The bis-organosilane used in the present work is completely redox-inactive, does not alter the electrode background potential window and contains no charged functional groups (like amino- or carboxylic groups), and thus is essentially indifferent to the immobilized protein. In this way, this approach can be seen as not only simple to use and efficient but also universal, and it should be compatible with any redox protein.

Experimental Section

Human neuroglobin (NGB) and GLB-12 from *C. elegans* were expressed in *E. coli* and isolated as described earlier.[4, 14] Inorganic salts, H_2SO_4 (96%), H_2O_2 (30%), 1,2-bis(trimethoxysilyl)ethane (BTMSE) and horse heart Cytochrome C (99.1% by SDS PAGE) were purchased from Sigma-Aldrich (Belgium). 6-Mercapto-1-hexanol (MH, >98.0 %) was from TCI Europe N.V. (Belgium).

The gold disks electrodes (BASi, West Lafayette, IN, USA) with a diameter of 1.6 mm were carefully polished initially using diamond of 3, 1 and 0.25 µm particle size (DP-Spray Struers, Ballerup, Denmark) in an alcohol-based lubricant (DP-lubricant Struers, Ballerup, Denmark), followed by a water based γ-alumina slurry of 50 nm particle size (SPI Supplies, West Chester, PA, USA). Before modification with MH, the electrodes were electrochemically treated in 0.5 M NaOH and 0.5 M H_2SO_4 until a repetitive and representative voltammogram of polycrystalline gold was achieved.[15] The roughness of the electrodes was assessed through the reduction peak of gold[16] and was found in the range of 1.2-1.4, which suggests that the electrode surface was essentially smooth.[16a] The electrodes were treated for 20 hours in MH (8 mM in H_2O). Prior the protein immobilization, the electrodes were washed in water, and background CV curves were recorded. Then, 1.5 µL of a mixture consisting of 10-20 µM protein and 10 mM BTMSE prepared in 50 mM KH_2PO_4 buffer (pH 7) was placed on the electrodes and dried at room temperature for 1 hour.

Cyclic voltammetry (CV) was conducted in a conventional three-electrode cell using the µAutolab III equipped with current integration module and supported by Nova 1.10 software (Metrohm-Autolab BV, the Netherlands). A saturated calomel electrode (SCE, Radiometer, Denmark, 0.248 V versus SCE at 20°C) and a glassy carbon rod were used as a reference and a counter electrode, respectively.

Acknowledgements

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Keywords: electron transfer • heme proteins • NGB • silane • globin

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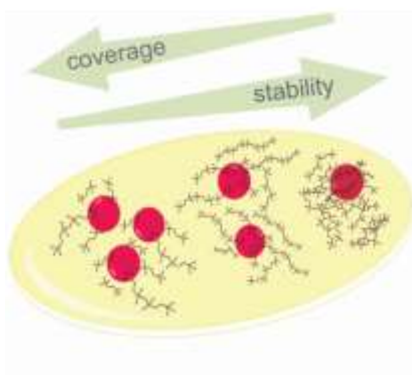
Table of Contents

COMMUNICATION

Silane keeps protein connected: Bis-organosilane (BTMSE) was applied as a fresh solution in a pH 7 phosphate buffer without use of an organic solvent and sol-gel preparation. A short aging period of 30 minutes before deposition on the electrodes was optimal for the immobilization of the protein leading to high coverage and excellent film stability.



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